Did Cholera Toxin Finally Get Caught?

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To orchestrate immune responses, pathogen-recognition receptors have evolved sophisticated strategies to monitor pathogenic processes. In this issue of *Cell Host & Microbe*, a study by Cho et al. reveals a mechanism of immune recognition that relies on the sensing of cholera toxin within the endoplasmic reticulum.

Over the last two decades, various mechanisms have been elucidated by which animal cells detect the presence of microbial pathogens and mount an immune response. These include Toll-like receptors, present at the cell surface and in endosomes to detect extracellular and endosomal microbial products, and receptors such as Nod-like and RIG-I-like receptors that ensure similar sensing in the cytosol. Recently, it was found that cells can even sense pathogen-induced alterations in cytoplasmic ion composition and in cytoskeleton organization and dynamics.

Yet some pathogen-derived products, in particular certain bacterial toxins, appear to remain undetected by the innate immune system, even though they enter the host cell to cause disease. One such particularly clever and sneaky toxin is cholera toxin (CT), the causative agent of the watery diarrhea that characterizes infection by the gram-negative bacterium *Vibrio cholerae* (Wernick et al., 2010). CT is an AB_5 toxin, where the A moiety carries the enzymatic activity and the homopentameric B moiety (CTB) is responsible for binding the toxin to the target cell surface and escorting it to the appropriate destination. In many toxins, the B subunit has the capacity to translocate the A subunit across a membrane, allowing it to reach its cytosolic targets. CTB acts instead by escorting CTA into the endoplasmic reticulum, the only site of the endomembrane system where protein transport across the membrane to the cytosol normally occurs. To reach this destination, CT has evolved, in a masterly fashion, to cheat its host, hiding its foreign origin and passing for a self-protein. The CTB pentamer binds to GM1 gangliosides at the cell surface, triggering an unconventional internalization route of sphingolipid trafficking that targets CT first to endosomes and then via retrograde transport to the Golgi and endoplasmic reticulum (ER).

One of the mechanisms by which eukaryotic cells localize soluble proteins within the ER lumen, such as Bip and other protein folding chaperones, is through the presence of a C-terminal KDEL sequence. This peptide motif is recognized by the KDEL receptor present in the early secretory pathway, which functions to capture KDEL bearing proteins as they "escape" the ER by bulk flow in secretory vesicles en route to the *cis* Golgi and recycle them back to the ER. Through the presence of a KDEL sequence at the C terminus of the A subunit, CT hijacks this ER retrieval pathway to localize within the ER lumen, at least long enough for the second coup de maître of CT coevolution to take place (Fujinaga et al., 2003; Lencer et al., 1995; Wernick et al., 2010).

Once inside the ER, the CT A subunit somehow masquerades as a terminally misfolded protein. It is recognized by the ER quality control machinery, unfolded with the help of ER chaperones/folding enzymes, and targeted to the ER-associated degradation (ERAD) pathway for retrotranslocation to the cytosol, basically co-opting the machinery for degradation of terminally misfolded endogenous proteins in the secretory pathway. Transport of misfolded proteins from the ER lumen to the cytosol during ERAD is normally followed by polyubiquitination and degradation by the proteasome. Yet, CT escapes ubiquitination, refolds into its enzymatically active conformation, and induces disease (Wernick et al., 2010). Thus, CT sneaks into the cell. It does not insert exogenous pores or protein conducting channels to breach cell membranes or otherwise damage the host cell. Rather, it poses successfully as a host cell protein, efficiently co-opting the normal cellular mechanisms for lipid trafficking and protein quality control in order to reach its goal.

Cho et al. now reveal how this so far thought to be "invisible" and ultimately successful toxin does not pass unnoticed (Cho et al., 2013). They find that CT is detected by a surveillance mechanism uniquely present within the lumen of the ER that subsequently triggers the production of proinflammatory cytokines such as interleukin- 6 (IL-6) and IL-8. Cytokine production in both mice and tissue culture is indeed observed with catalytically inactive and retrotranslocation-deficient CTA mutants, excluding the involvement of cytosolic pattern-recognition receptors.

The ER plays a central role in maintaining cellular homeostasis. It serves a variety of key functions, including the production of virtually all membrane and secreted proteins. To cope with physiological and pathological conditions that challenge its functions, the ER displays a remarkable capacity to adapt and restore homeostasis. In particular, the pathological or stress-induced accumulation of unfolded/misfolded proteins in the lumen is sensed by transmembrane signaling molecules that trigger a transcriptional program known as the unfolded protein response (UPR) (Walter and Ron, 2011). IRE1α is the best-studied and most-conserved branch of the UPR, the only one present in lower eukaryotes. It detects unfolded proteins within the ER lumen and signals via an extension in the cytosol that contains two functional units: a protein kinase that serves as an oligomerization platform and an endoribonuclease that targets cellular RNAs. IRE1α activation triggers the expression of an active XBP1 transcription factor by

removing a short sequence in the *XBP1* messenger RNA (mRNA) that changes its translational reading frame.

Cho et al. found that CT-induced cytokine production depends on the IRE1α ribonuclease activity, while not requiring the other UPR ER sensors. Surprisingly, it does not involve XBP1, the best-characterized downstream IRE1α effector. In addition to XBP1 activation, IRE1α can degrade a pool of mRNAs and microRNAs through a process known as regulated IRE1α-dependent decay (RIDD) (Hetz et al., 2011; Hollien and Weissman, 2006). It shares this property with RNaseL, a cytosolic endonuclease that evolved from IRE1α to operate in host defense against invading viruses. Small self-RNA fragments generated by RNaseL in response to invading viruses can activate the antiviral innate immune sensor RIG-I (Malathi et al., 2007). Cho et al. found that self-RNAs generated by IRE1α via CT-activated RIDD also engage the RIG-I pathway to activate the NF-κB pathway, leading to cytokine release—this time in response to a microbial product entering the ER (Figure 1).

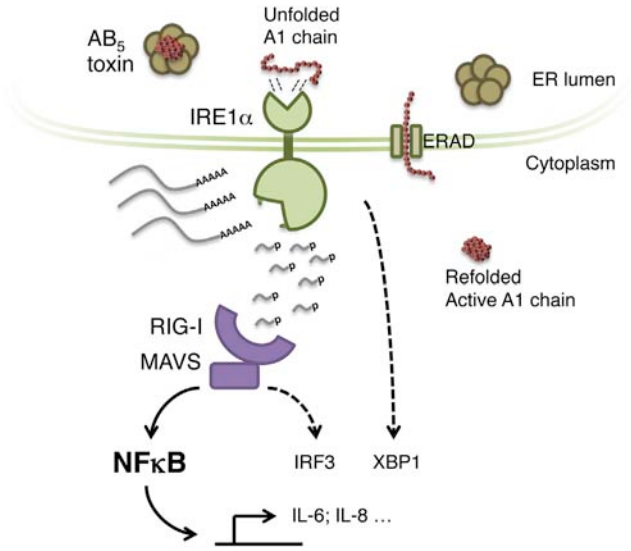

Figure 1. Activation IRE1α by CT within the ER Triggers NF-κB and Cytokine Release

The CT that reaches the lumen of the ER is formed by five units of its B subunit and one subunit A. For translocation of the active A1 chain in the cytosol, the A1 chain is first unfolded and then retrotranslocated via the ERAD machinery in the cytoplasm. In the meantime, the ER-anchored stress sensor IRE1α detects the unfolded A1 chain within the ER and is activated. IRE1α harbors an RNase activity that on one hand converts XBP1 mRNA in its active form and on the other hand degrades cellular RNAs. IRE1-processed RNAs are then detected by the RIG-I pathogen recognition receptor that recruits its adaptor MAVS and activates NF-κB and IRF3 transcription factors. The activation of NF-κB leads to the production of inflammatory cytokines such as IL-8 and IL-6.

Activation of RIG-I in response to viral infection triggers two distinct pathways: the production of type I interferon (IFN) via the transcription factors IRF3 and IRF7 and the production of inflammatory cytokines via NF-κB. CT was found to trigger both branches, but despite IRF3 activation, the resulting type I production is modest compared to the induction of inflammatory cytokines. These data are intriguing and could reflect the specific nature of the RNA fragments that are generated by RIDD in response to CT. These fragments do not harbor the 5′ triphosphates present in most viral RIG-I agonists. They may therefore orchestrate a specific RIG-I response skewed toward NF-κB and inflammatory cytokine production. Alternatively, other signals stemming from IRE1α activation may impact on type I IFN production in epithelial cells. One possible mechanism could be the activation of XBP1, which binds the type I IFN promoter in macrophages (Zeng et al., 2010).

The study by Cho et al. highlights the existence of a microbial surveillance pathway in the lumen of the ER. It also raises many questions regarding the transcriptional response, as mentioned above, and the mechanisms of recognition. Classically, activation of the UPR is thought to occur when the ER content of unfolded/misfolded proteins reaches a given threshold. Analysis of the yeast IRE1α structure suggests that the luminal domain directly binds unfolded protein segments, much like MHC complexes bind antigenic peptides, and this leads to oligomerization and activation of IRE1α (Korennykh and Walter, 2012). It is, however, well established that only very few molecules of CT actually reach the ER. It is therefore unlikely that the threshold of unfolded proteins tolerated by the ER is reached during CT intoxication, suggesting that activation occurs through a more-specific recognition of CTA by IRE1α. In line with this hypothesis, Cho et al. show, using a peptide tiling array and coimmunoprecipitation, that two surface-exposed sequences in CTA can bind IRE1α. This binding might have the ability to activate IRE1α, at concentrations far below that required by unfolded proteins that typify the canonical ER stress response. The notion that specific binding might activate IRE1α is slightly hampered by the observation of Cho et al. that other pathogens/toxins, namely Shiga toxin (ST) and the SV40 virus, also activate the IRE1α-RIG-I axis to trigger cytokine production. Much like CT, ST and SV40 bind gangliosides at the cell surface to force their way into the cells and are transported via the retrograde pathway to the ER, where they subsequently cross the membrane to reach the cytosol. The authors propose that IRE1α may have evolved to sense the structurally diverse CT, ST, and SV40 capsid proteins by somehow sensing common features required for unfolding and retrotranslocation to the cytosol. Alternatively, the toxins and viruses might have independently evolved, driven by as-yet-unknown selective forces, to bind IRE1α in a manner that triggers activation; or perhaps activation occurs through a more-generic binding event that mimics the unfolded protein response.

The discovery that the RIDD pathway stemming from danger surveillance within the ER can engage innate immune receptors for virus invasion of the cytosol such as RIG-I to trigger NF-κB activation emphasizes the mechanistic and evolutionary connection between cellular stress responses and innate immune pathways. Infections and diseases, such as cystic fibrosis, inflammatory bowel disease, atherosclerosis, and type 2 diabetes, display features characteristic of both ER stress and inflammation, including the activation of IRE1α, neutrophil, and macrophage infiltration. It will be particularly important to address the significance and physiological relevance of IRE1α-mediated RIDD and

RIG-I activation, revealed by Cho et al., in these pathologies. Such studies will undoubtedly shed new light on the role of the ER as a stress sensor that regulates health, inflammation, and susceptibility to infections.

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The Oral Commensal Microbiota Bites Back through Nod1

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The mechanisms through which commensal bacterial populations cause inflammatory disease when shifted to dysbiotic community structures are poorly understood. Jiao et al. (2013) demonstrate that, in the case of inflammatory disease in the mouth, stimulation of the intracellular pattern recognition receptor Nod1 is a critical determinant.

Periodontal disease is one of the most prevalent chronic inflammatory diseases of humans and is characterized clinically by the irreversible destruction of the bony apparatus that secures the teeth in the mouth. A hallmark of the disease is a dysbiotic periodontal microbiota, which has been extensively investigated by cultural analysis over the last 50 years and more recently by next-generation sequence analysis of the complement of 16S ribosomal RNA genes in healthy compared to diseased-associated microbiota (Wade, 2011). The rearrangement of the normally benign commensal microbial populations on the surface of the teeth provides a sustained antigenic challenge to the adjacent soft tissues, which, in susceptible individuals, leads to a deregulated inflammatory response and ultimately resorption of the underlying hard tissue. The precise mechanisms through which the dysbiotic periodontal microbiota induces deregulated inflammation are unknown. However, the end result, characterized by large elevations in neutrophil *trans*-migration into the tissues and the differentiation and activation of bone-resorbing osteoclasts through RANK ligand expression by $CD4^+$ T cells, has been well established in both human and animal model systems (Darveau, 2010).

Two mouse models of periodontal bone loss have been customarily employed to interrogate the pathogenesis of the disease. In the first, a bacterium frequently associated with disease in humans, for example *Porphyromonas gingivalis*, is inoculated into the mouth of experimental mice, leading to the induction of bone loss some 6 weeks postcolonization. Hajishengallis et al. (2011) demonstrated that bone loss in this model was dependent upon the presence of a commensal microbiota: Colonization of germ-free animals with *P. gingivalis* results in no tissue damage. Furthermore, analysis of the microbiota of conventionally reared mice challenged with *P. gingivalis* demonstrated that introduction of this organism caused a significant increase in the total oral microbial load and dysbiosis of the overall microbial community structure. These data indicated that the presence of *P. gingivalis*, even at a low abundance, manipulates the commensal microbiota into a dysbiotic community that leads to the development of disease.

In the second model of disease, a silk ligature is placed around selected molar teeth in order to provide a site for increased microbial accumulation and tissue irritation. Just 10 days following ligature placement, periodontal bone loss is observed, indicative of an acute response to this intervention. In the current issue, Jiao et al. (2013) have employed this model system in knockout mice to determine which elements of the pattern recognition receptor system of the host are required to elicit this destructive response. They demonstrate that Nod1 (*n*ucleotide-binding *o*ligomerization *d*omain-containing protein *1*), a